Activation of complement by an IgG molecule without a genetic hinge

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The hinge region links the two Fab arms to the Fc portion of the IgG molecule. It mediates flexibility to the molecule and serves as a connecting structure between the two heavy chains. In addition it provides space between the Fab and Fc parts. All three properties have been proposed to be important for the ability of IgG to initiate complement activation leading to complement-mediated cell lysis (CML)[1]. Here we report the construction of a hinge-deleted mouse–human chimaeric IgG3 molecule with specificity for the hapten NIP (3-iodo-4-hydroxy-5-nitro-phenacetyl), HM-1. HM-1 lacks the genetic hinge, but has an introduced cysteine between Ala 231 (EU numbering) and Pro 232 in the lower hinge encoded by the C_H2 exon. The introduced cysteine forms a disulphide bond between the two heavy chains of the molecule. In CML, HM-1 shows a greater activity than IgG3 wild type. This is the first time an IgG molecule without a genetic hinge has been found to be active in CML. We conclude that the hinge functioning as a spacer is not a prerequisite for complement activation. Rather, its major role seems to be to connect the heavy chains to each other in the amino-terminal part of C_H2. Because HM-1 is expected to have low Fab–Fc flexibility, this molecular feature is probably of no importance for complement activation.

We have produced a hinge-deleted IgG3 molecule, m0, by removing all four hinge exons from the human Cγ3 gene (Fig. 1). This molecule has no disulphide bonds between the heavy chains (Fig. 2*a*). On a nonreducing sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gel a large proportion of m0 molecules run as heavy–light (H–L) half molecules (Fig. 3*a*). In the mutant HM-1 (Fig. 2*b*), a cysteine was introduced between Ala 231 and Pro 232 in m0 by *in vitro* mutagenesis. SDS–PAGE analysis of HM-1 showed no presence of H–L half molecules (Fig. 3*a*). As m0 and HM-1 are identical except for the introduced cysteine, it is likely that the heavy chains are connected through a disulphide bond in this position. HM-1 was active in CML, and even more active than IgG3 wild type under different antigen concentrations and antigen patchiness, whereas m0 lacked CML activity completely (Fig. 4). The mutant m15, which was made by deleting three hinge exons from the Cγ3 gene and therefore contains a hinge region of 15 amino acids encoded by one remaining hinge exon only[2], showed CML activity comparable to HM-1 (Fig. 4). In these CML assays, antigen was introduced to the target cell surface, varying both the antigen concentration and antigen patchiness. Regardless of whether the target cells had a low, medium or high antigen concentration and antigen patchiness, both HM-1 and m15 initiated lysis more effectively than normal IgG3.

Naturally occurring IgG molecules with rigid and short hinges show reduced complement activation ability[3]. It has been suggested that this phenomenon is due to the two Fab arms covering the binding site for the first component of the complement system, C1q, on the C_H2 domain of Fc[1], involving the amino acids Glu 318, Lys 320 and Lys 322[4]. Segmental flexibility between Fab arms and Fc (reviewed in ref. 5) depends on the length of the upper hinge[3] which stretches from the end of the C_H1 to the first inter-heavy-chain disulphide bond in the hinge region[6]. We and others[2,7–9] have constructed artificial chimaeric mouse–human IgGs where the hinge region of active

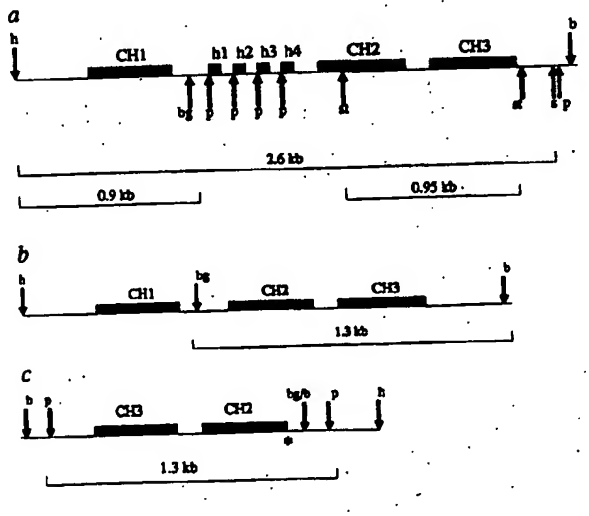

FIG. 1 Restriction maps of immunoglobulin gene constructs. *a*, Restriction map of the human Cγ3 gene. Exons are shown as boxes, h, *Hin*dIII, bg, *Bgl*I, p, *Pst*I, st, *Sty*I, s, *Sph*I and b, *Bam*HI. *Pst*I and *Bam*HI sites outside the 2.6 kilobase (kb) gene construct resides in the pUC19 polylinker. *b*, The m0 gene construct. *c*, C_H1–C_H3 fragment as it is inserted into the M13mp18 polylinker. An asterisk marks the site of the mutation. METHODS. A 2.6-kb *Hin*dIII–*Sph*I fragment containing the full-length gene of human Cγ3 (G3m(b^0) allotype) (*a*) was subcloned into pUC19[2]. The Cγ3 gene was partially digested with *Pst*I. The resulting gene construct obtained, m15, contained only the h4 hinge exon. m15 was linearized by digestion with *Bgl*II. The linearized plasmid was then subjected to exonuclease III and S1 nuclease digestion. After a Klenow end-filling reaction, *Bgl*II linkers were added before ligation. A plasmid that had thereby lost its h4 exon had also lost part of the C_H1 exon. The gene construct was digested with *Hin*dIII and *Bgl*II and combined with a complete C_H1 exon on a *Hin*dIII–*Bgl*II fragment to give the m0 gene construct (*b*). The template for the mutagenesis was a 1.3kb *Bam*HI–*Bgl*II fragment containing the C_H3 and C_H2 exons. This fragment was subcloned into the *Bam*HI site of the polylinker of M13mp18 (*c*). *In vitro* mutagenesis was as described[10]. The mutagenesis oligonucleotide primer was as follows: 5'catctcttcctcagcctgccctgaactcc3'. Nucleotides coding for cysteine (underlined) were thus inserted into the C_H2 exon and the mutant sequence was verified by automatic DNA sequencing by the Sanger dideoxy chain-termination method[20]. Only the sequenced part of the C_H2 exon encoding the N-terminal region was used. The rest of the C_H2–C_H3 fragment was exchanged with a wild-type C_H2–C_H3 *Sty*I-fragment of 950 base pairs (bp) (*a*). The mutant 1.3-kb C_H2–C_H3 *Pst*I fragment (*c*), was then cloned into a pUC19 vector containing the 0.9-kb C_H1 *Hin*dIII–*Pst*I fragment. The mutant Cγ3 gene construct was finally cloned into the pSV2gptV_{NP} shuttle vector[21] as a 2.2-kb *Hin*dIII–*Bam*HI fragment. Transfection was done by electroporation of J558L cells as described[7,22]. The V_{NP} segment of the pSV2gpt vector expresses the V_H-region characteristic of a λ_1 light-chain-bearing mouse antibody that recognizes the hapten NIP[23]. J558L does not produce an immunoglobulin heavy chain but produces a λ_1 light chain. The mutant chimaeric antibodies produced by the transfectomas thus have specificity for the hapten NIP. Clones were selected in medium containing mycophenolic acid and xanthine, screened for antibody production by ELISA[2] and subjected to limiting dilution.

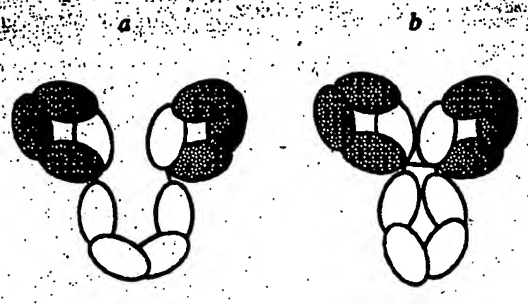

FIG. 2 Models of chimaeric mouse–human mutant IgG. *a*, Model of m0 produced as H–L half molecules. Shaded domains are murine and open domains are human. The heavy chains are not connected as the genetic hinge is deleted. The resulting conformation of the molecule is that of an open structure as there are noncovalent interactions between C_H3 domains[7]. *b*, Model of HM-1. The heavy chains are covalently linked by a disulphide bond provided by the inserted cysteine as the second amino acid of the lower hinge.

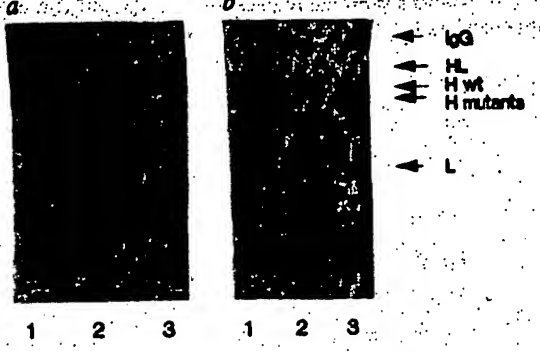

FIG. 3 SDS–PAGE gel (12%). The lanes contain: 1, m0 mutant; 2, IgG3 wild type; and 3, HM-1 mutant. *a*, Without dithiothreitol (DTT) treatment. *b*, Treated with 10mM DTT. IgG, H_2L_2; HL, heavy and light chain; H wt, wild type heavy chain monomers; H mutants, mutant heavy-chain monomers; L, light-chain monomers.
METHODS. Antibodies were isolated from 1 litre of cell culture supernatants by affinity chromatography on an NIP-Sepharose column and eluted with the hapten NIP as described[2], then analysed by SDS–PAGE on a 12% gel as described[24].

IgG molecules has been made short and rigid by removing amino acids in the N-terminal region. These molecules lost none of their complement activation potential. Thus, segmental flexibility has been shown to be of no importance for CML. But the hinge region seems to be necessary for complement activation because all IgG mutants without the genetic hinge so far are inactive[7,10,11]. The middle, or core hinge, contains cysteines mediating disulphide bonds between the heavy chains and a rigid polyproline core which separates Fab from Fc[12,13]. In the Dob and Mcg molecules, both without genetic hinge, the light chains are disulphide-bonded to each other, and the crystal structures of these molecules show that the distance between Fab and the C_H2 domains is very short[14,15]. It has therefore been proposed that the hinge may serve to create space between the Fabs and the Fc part of the molecule, allowing Clq to interact with its binding site on C_H2. Because HM-1 lacks the genetic hinge region, the distance between the Fab arms and Fc must be short. Therefore, it seems likely that the ability of the hinge to provide space between the Fab arms and Fc is not necessary for complement activation. Neither does HM-1 have an upper hinge, as there is only a single amino acid between the end of C_H1 and the first inter-heavy-chain disulphide bond, namely Ala 231. This amino acid belongs to the lower hinge encoded by the C_H2 exon. Segmental flexibility between the Fab arms and Fc is therefore not necessary for complement activation. Rather we observed that the presumably inflexible HM-1 molecule activated complement better than the corresponding IgG3 wild-type molecule regardless of antigen concentration and antigen patchiness. We also observed this phenomenon when testing m15, which has a short and rigid hinge[7,16]. It seems as though the long and flexible hinge of IgG3 wild type reduces its CML potential. Clq binding to rigid molecules may be energetically favourable compared to the flexible IgG3 molecule, as the loss of entropy on complex formation is greater for a flexible molecule than for a rigid one.

Our mutant HM-1 and the proteins Dob and Mcg all consist of two heavy chains and two light chains (H_2L_2). But unlike HM-1, both Dob and Mcg have the two H–L half molecules disulphide-bonded through linking of the light chains[15,14] and neither can complement fixate[10,11]. Because the HM-1 molecule has CML activity, there must be conformational differences between these proteins regarding access to the Clq binding site. A clue to

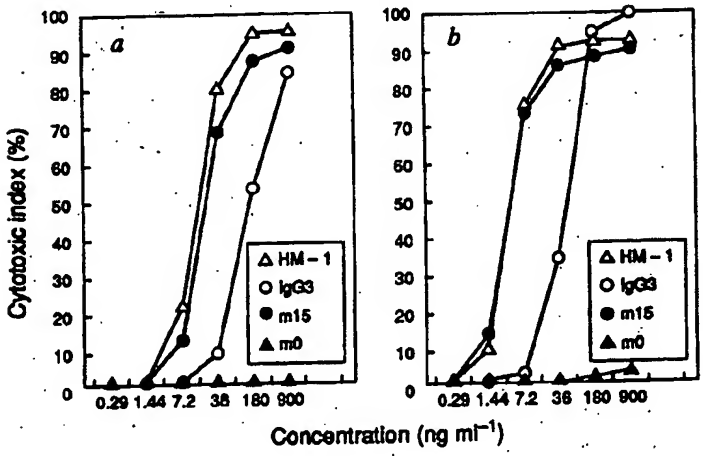

FIG. 4 CML activity induced by NIP-specific antibodies measured in a ^{51}Cr-release assay. The target cells were sheep red blood cells (SRBC), and the hapten NIP was introduced on the cell surface conjugated to anti-SRBC Fab fragments. For 1×10^8 SRBC a total of either: *a*, low amount, 80 ng NIP with NIP_4Fab; or *b*, high amount, 2,000 ng NIP with $NIP_{60}Fab$. HM-1, hinge-deleted IgG3 with an introduced cysteine; m0, hinge-deleted IgG3; m15, IgG3 with genetic hinge of 15 amino acids; IgG3, IgG3 wild type.
METHODS. The CML assay was done by varying antigen concentration and antigen patchiness as described previously[7]. Briefly, target cells, ^{51}Cr-SRBC, were allowed to react with NIP-conjugated rabbit anti-SRBC Fab' fragments. Serial dilutions of chimaeric antibodies were then added to the target cell suspension and human serum was used as the complement source. The cytotoxic index (CI) was calculated according to the formula:

$$\%\,CI = \frac{\text{c.p.m. (test)} - \text{c.p.m. (spontaneous)} \times 100}{\text{c.p.m. (max)} - \text{c.p.m. (spontaneous)}}$$

these differences is probably the disulphide bond between the light chains in Dob and Mcg, which is not present in the HM-1 molecule.

Hinge-deleted molecules such as m0, lack disulphide bonds between the heavy chains. The C_H2 domains will therefore drift apart because there are no transinteractions between these domains[17,18] (Fig. 2*a*). As a result, the Clq-binding sites on the two C_H2 domains will be dislocated and may even be distorted. Because Clq is multivalent in its binding to IgG, tight cooperative binding between IgG and Clq may be impossible for the m0 molecule. The only difference between m0 and HM-1 is in the ability of HM-1 to form a disulphide bond between its heavy chains. Thus in complement activation the major role of the hinge seems to be covalent linking of the heavy chains.

It has been difficult to crystallize intact active IgG molecules for X-ray diffraction analysis because of the flexible hinge region. The mutant HM-1 is likely to be rigid, therefore it may be a good candidate for X-ray analysis of an intact, functionally active IgG molecule.

Note added in proof: We have recently shown that HM-1 promotes phagocytosis mediated by FcRI with high efficiency, but m0 is negative[25]. Thus, bridging of the heavy chains is also a prerequisite for efficient FcR signalling, whereas Fab arm flexibility and distance between Fab and Fc seem not to be necessary. □

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Segmental organization of embryonic diencephalon

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The diencephalon is a complex integration centre and intricate relay station of the vertebrate brain[1–3]. Its development involves the generation of great cellular diversity and neuronal specificity. We report here that it becomes organized in steps, through a stereotyped sequence of neuromeric subdivisions. Diencephalic neuromeres define four cellular domains (D1–D4) that can be followed throughout development, each unit contributing to a well defined part of the adult structural pattern. We propose that the segmental identity of each diencephalic unit is specified by a unique combination of genes[4–13], maintained by polyclonal cell lineage restrictions. A comparison of vertebrate and arthropod development suggests that the basic principles that control anterior axial patterning and set up neuronal specificity in the embryonic central nervous system are highly conserved in evolution.

Many morphological studies have been done[14–21] to determine the basic units of pattern formation in the forebrain, but they have come to irreconcilable conclusions. We have now studied the development of the embryonic diencephalon of the chick embryo by scanning electron microscopy (SEM). A pattern of transverse furrows and ridges is seen, repeated along the anteroposterior axis of the third ventricle (Fig. 1*a*). Four regions can be distinguished, which we designate as neuromeres D1–D4, numbered in anterior-to-posterior sequence. The borders between neuromeres appear as ridges, subdividing the otherwise smooth relief of the third ventricle. They form in precise temporal sequence (D4/M: stage-12 (ref. 22); D1/D2:

FIG. 1 Morphology of diencephalic neuromeres and alternating patterns revealed by antibodies. *a*, SEM of stage-24[22] chick embryo in medial view. *b*, NCAM is accumulated at neuromere boundaries: the border between D1 and D2 stains intensely. Whole mount of stage-24[22] embryo. Acetylcholinesterase activity (*c*) and peanut agglutinin (*d*) reveal an alternating pattern of staining: even-numbered neuromeres show high levels of activity. In D4, the development of the early tract of the posterior commissure (TPC axons) is seen (*c*), restricted at both anterior and posterior boundaries of D4. Whole mount of stage-25[22] embryo. D1–D4, diencephalic neuromeres. T, telencephalon. M, mesencephalon.
METHODS. For SEM, chick embryos at stages 12–39[22] were explanted in PBS, pH 7.4 at 4°C and the brain bisected. They were fixed for 1–3 h in 2% paraformaldehyde, 2.5% glutaraldehyde and 0.025% $CaCl_2$ in 0.1 M cacodylate buffer (pH 7.4). After several washes in this buffer, they were incubated for 1 h in 2% OsO_4, washed in cacodylate buffer, dehydrated, transferred to acetone and critical-point dried overnight. Specimens were mounted on stubs, sputter coated (Nanotech SEM Prep 2, 4 min) and viewed with a Philips SEM 515. Antibody, cholinesterase and lectin histochemistry were done by previously described techniques[23–25]. Anti-NCAM antibody was a gift of Dr U. Rutishauser.

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